



## Histological characteristics and markers of proliferation and differentiation in rat brain with experimental glioma

Histološke karakteristike i markeri proliferacije i diferencijacije u mozgu pacova sa eksperimentalnim gliomom

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### Abstract

**Background/Aim.** The cell line C6 is a continuous cell line of rat glioma and, as a transplantable line, is frequently used for induction into *in vivo* model of primary brain tumor. It is believed that, pursuant to its histological traits and biological behavior, this experimental tumor corresponds to human anaplastic astrocytoma of grade II/III, which is characterized by proliferative and invasive potency, and marked cell differentiation. The aim of this study was to determine macroscopic analysis of rat brain with implanted tumor during tumorigenesis, histological features of tumor cells of induced brain tumor and markers of proliferation (proliferation cell nuclear antigen – PCNA, cytokeratin – CK 19) and differentiation (glial fibrillary acidic protein – GFAP) in rat brain with implanted tumor. **Methods.** To determine histological structure of the brain with implanted C6 cells, we used brain sections stained for hematoxylin-eosin or kresyl violet, whereas other sections were immunohistochemically stained for GFAP, CK 19 and PCNA. **Results.** A statistically significant difference in weights of the left and right brain hemispheres with implanted tumors during tumorigenesis in as soon as 7 days from the day of inducing tumors was revealed. The tumor was of cellular type, with distinct pleomorphism of cells and frequent hyperchromasia of the nucleus. Immunohistochemical staining for PCNA revealed a significant number of positive cells on the days 7, 14 and 21 day following the implantation of C6 cells. CK 19 positive cells were present in both brain hemispheres, and numerous GFAP positive astrocytes were found around the puncture lesion. **Conclusions.** Within the experimental conditions of the present research, C6 glioma did not demonstrate any relevant deviations concerning development, clinical symptomatology and macroscopic anatomy relative to those already described in the literature.

### Key words:

glioma; rats; disease models, animal; immunohistochemistry.

### Apstrakt

**Uvod/Cilj.** Čelijska linija C6 predstavlja kontinuiranu čelijsku liniju glioma pacova i kao transplantabilna linija, često se koristi za indukciju *in vivo* modela primarnog moždanog tumora. Smatra se da po histološkim osobinama ili biološkom ponašanju ovaj eksperimentalni tumor odgovara humanom anaplastičnom astrocitomu gradusa II/III, koji karakteriše proliferativni i invazivni potencijal, kao i izražena čelijska diferencijacija. Cilj ovog rada bio je da se odrede: makroskopska analiza mozgovog pacova sa implantiranim tumorom u toku tumorogeneze, histološke karakteristike tumorskih ćelija indukovano mogdanog tumora i markeri proliferacije (proliferativni čelijski nuklearni antigen – PCNA, citokeratin – CK 19) i diferencijacije (glialni fibrilarni kiseli protein – GFAP) u mozgu pacova sa implantiranim tumorom. **Metode.** Za određivanje histološke građe mozga sa implantiranim C6 ćelijama korišćeni su iseći mozga obojeni hematoksilin-eozinom ili krezil violet bojom, dok su drugi iseći imunohistohemijski obojeni na GFAP, citokeratin 19 i PCNA. **Rezultati.** Utvrđeno je da postoji statistički značajna razlika u težini između leve i desne hemisfere mozgovog sa implantiranim tumorima u toku tumorogeneze već 7 dana od indukcije tumora. Tumor je bio celularnog tipa, sa izraženim pleomorfizmom ćelija i čestom hiperhromazijom nukleusa. Imunohistohemijsko bojenje na PCNA pokazalo je veliki broj pozitivnih ćelija posle 7, 14, i 21 dan od implantacije C6 ćelija. Ćelije pozitivne na CK 19 bile su prisutne u obe hemisfere mozga, a brojni GFAP pozitivni astrociti nađeni su oko mesta ubodne lezije. **Zaključak.** U eksperimentalnim uslovima ove studije gliom C6 nije pokazao značajnija odstupanja u smislu razvoja, kliničke simptomatologije i makroskopske anatomije od one koja je već opisana u literaturi.

### Ključne reči:

gliom; pacovi; bolest, modeli na životinjama; imunohistohemija.

## Introduction

One of the most frequently used tumor cell lines *in vitro* in neurobiology are C6 cells. The C6 cell line is a continuous cell line of rat glioma, which has been originally induced in Wistar rats by intravenous application of N-methyl nitrosourea<sup>1</sup>. Given that it is also a transplantable line, it is frequently used for *in vivo* induction of primary brain tumor model<sup>2</sup>. It is believed that, pursuant to its histological traits and biological behavior, this experimental tumor corresponds to human anaplastic astrocytoma of grade II/III<sup>3</sup>.

Clarification of biochemical pathways for the progression of cell cycle made it possible to identify the PCNA antigen (proliferating cell nuclear antigen), which made a practical tool as a tumor proliferation marker. The PCNA, a member of the cyclin family, is a nuclear protein which attaches to DNA delta polymerase and is necessary for replication of DNA<sup>4</sup>. Its presence is related to late G1 and S phases of cell cycle. Anti-PCNA antibodies are commercially available, and used to determine the proliferative potential of CNS tumor<sup>5</sup>. This antigen proved to be a more reliable and accurate marker of tumor cell proliferation relative to the mitotic index.

Cytokeratin 19 (CK 19) is an acidic protein that makes a part of epithelial cell structure. It contributes to the cell resistance, transduction of signals and regulation of cell migration and invasion<sup>6</sup>. As an intermediate filament excreted by epithelial cells, it is used as a marker for the differentiation of epithelial cells.

Glial fibrillary acidic protein (GFAP) is the key constituent of intermediate filaments with normal, reactive and neoplastic astrocytes. It is considered that the expression of GFAP in astrocytoma is in correlation with cell differentiation, and in inverse relation with proliferative potential<sup>7</sup>. The exception is protoplasmic astrocytoma, which displays either a minimal GFAP immunoreactivity or none at all<sup>8</sup>. Immunohistochemical detection of GFAP is vital in neuropathological research of astrocytoma<sup>9</sup>.

The experience so far tells us that when compared with similar experimental models C6 cells grow more homogeneous intracerebrally and imitate the growth of human glioma to a greater extent<sup>10</sup>, which was the basis for decision to monitor the said markers in the experimental rat glioma.

The aim of the research was to determine: macroscopic analysis of brains having implanted tumor during the tumorigenesis; histological traits of tumor cells of an induced brain tumor (animal model); PCNA, CK 19 and GFAP in rat brain having implanted tumor.

## Methods

C6 cells were used to induce experimental brain tumor. C6 cells were maintained in culture on nutrient medium, Dulbecco Modified Eagle Medium (DMEM), with added 10% v/v inactivated fetal calf serum (FCS) and mixture of antibiotics (penicilin-100 U/mL, streptomycin-100 µg/mL and amphotericin B-25 µg/mL). Flasks with cells were kept in humid environment with present 5% CO<sub>2</sub> at 37°C. In order to prepare cells for cerebral implantation, they were submit-

ted to trypsinization, centrifugation, rinsing and re-suspending in phosphate buffered saline (PBS).

Cerebral implantation of C6 cells was performed on Wistar male rats weighing on average 242 grams. They were firstly anesthetized by intraperitoneal application of sodium thiopental (50 mg/kg) and thereafter fixed in place on a wooden platform in the proper position. Next, the skin of head was depilated and disinfected. A scalpel was used for skin incision and subcutaneous tissue along the midline, in anteroposterior direction, for 1.5–2 cm. After removing the periosteum, craniotomy was performed using a dental drill, in the right-hand frontoparietal region of the skull, 4 mm to the right and 2–3 mm above the coronary satura. Using the Hamilton syringe, suspension of C6 cells ( $4 \times 10^6$  cells/10 nL PBS) was injected into the right brain hemisphere to the depth of 5 mm. Thereafter, the incision was shut by suture, and animals were placed into individual cages to recover. It took 21–25 days to develop the tumor in animals.

The second group of animals underwent the identical procedure, except that instead of C6 cells suspension, they were injected the same volume of media for making suspension (the group of ostensibly operated animals). Both groups of animals were nourished pursuant to standard hygienic and dietary regime up to the beginning of experiment.

After a light ether anesthesia, the animals were sacrificed by means of decapitation; the brains were carefully taken out and examined for macroscopic presence of tumor. The hemispheres were divided, weighed separately, and subjected to further procedure.

After sacrificing animals with implanted C6 cells, their brains were taken for histological analysis. The brain tissue was fixed in 4% paraformaldehyde (in phosphate puffer, pH 7,4) for 2–3 days at 4°C, and thereafter was performed cryoprotection in 30% Sukrozi and PBS for 2–3 days at 4°C. Then the tissue was sliced by cryotome (Reichert) at the temperature of -25°C. The section thick 14 nm were mounted on the previously jelly-coated microscopic plates and stored at -20°C. The sections were stained for hematoxylin-eosin or kresyl violet in order to visualize the overall histological structure of brain implanted with C6 cells, whereas other ones were immunohistochemically stained for GFAP, CK 19 and PCNA.

The ABC (avidin-biotin-peroxidase rena complex) method<sup>11</sup> was used to determine immunoreactivity as follows: rinsing the sections in PBS; blocking the endogen peroxidase by incubation of sections in 0.3% hydrogen peroxide in methanol; incubation with 20% normal goat serum to block the non-specified associating of secondary antibodies; incubation with solution of primary antibodies (polyclonal anti-GFAP ICN Pharmaceuticals 1:500; monoclonal anti-CK 19 ICN Pharmaceuticals 1:100; monoclonal anti-PCNA Boehringer Mannheim 1:100) in PBS with 1% BSA (bovine serum albumin), 1h; incubation with appropriate secondary antibody conjugated by biotin, 1h; incubation with avidin-biotin-peroxidase complex, 30 min; and stained reaction - diaminobenzidine (DAB)-H<sub>2</sub>O<sub>2</sub>, 5 min.

In between all incubations the rinsing in PBS was performed. For methodological control sections incubated with-

out primary antibodies were used. All reactions took place at room temperature.

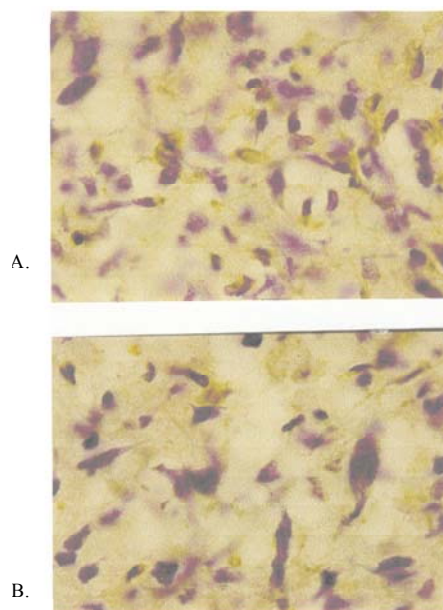
The DMEM nutrient medium, FCS, ready-made mixtures of antibiotics and antimycotics, solution for trypsinization, all other standard chemicals for maintaining cell cultures, and plastic disposable containers, were given by ICN Pharmaceuticals, Costa Mesa, USA. The C6 rat glioma cells were given by Prof. Dr. Stukalov (Institute for Molecular Genetics, Moscow, Russia).

Male Wistar rats were bred in the Vivarium of the Center for Biomedical Research, ICN Galenika Institute.

## Results

### Macroscopic analysis of tumor

The presence of tumor was often accompanied by edema, although the tumor itself was not always macroscopically visible. Macroscopic tumors were only visible in 20% of cases, as a bump in the right hemisphere with a 2 mm diameter. Measuring of the right and left hemispheres of brains with implanted tumors during the tumorigenesis revealed a statistically significant difference in weight between the left and the right hemispheres in as soon as 7 days after the tumor incubation event (Table 1).



**Fig. 1 – Immunohistochemical staining for proliferating cell nuclear antigen (PCNA), ABC method and contrast staining for krezyl-violet.**

A) PCNA positive nuclei of C6 cells in the right brain hemisphere 14 days after the implantation ( $\times 1,000$ ). B) The number of PCNA – positive C6 cells in the left hemisphere is substantially lower ( $\times 1,000$ ).

**Table 1**

**Weights of the right and left hemispheres of rat brains with C6 tumor**

Days after implanting C6 cells	Brain weights (mg)	
	right hemisphere	left hemisphere
7	702.2 $\pm$ 71.4*	603.7 $\pm$ 20.2
14	620.0 $\pm$ 36.6	622.6 $\pm$ 82.5
21	592.6 $\pm$ 33.7	598.0 $\pm$ 20.2

The results are given as an average value  $\pm$  standard deviation; \*  $p < 0.01$ .

### Microscopic structure of C6 rat glioma

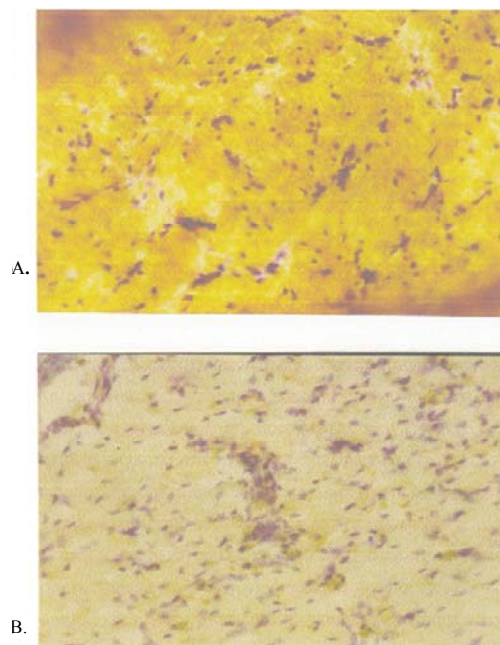
Histological preparations stained by hematoxylin-eosin had a clear place of puncture lesion in the right brain hemisphere, however the tumor mass itself could rarely be noticed, due the tendency of C6 cells' to infiltrative growth. The tumor was of cellular type, with a marked pleomorphism of cells and a frequent hyperchromasia of the nucleus.

Immunohistochemical staining for PCNA revealed numerous PCNA positive cells in the right brain hemisphere migrating along the white matter, and also appearing in the left hemisphere (Figure 1).

The proliferation rate index (number of positive C6 cells relative to all implanted cells) could not be determined, so that the monitoring of this index over time was not possible. However, a significant number of positive cells were noticed on the days 7, 14, and 21 after the implementation of C6 cells, meaning that these cells are still proliferating beyond the day 21.

Immunohistochemical staining for cytokeratin 19 revealed numerous CK 19 positive cells in both hemispheres of the brain (Figure 2).

The preliminary results showed that all C6 cells *in vitro* were CK 19 positive. A significantly higher number of cells were found in the right hemisphere, where C6 cells had been

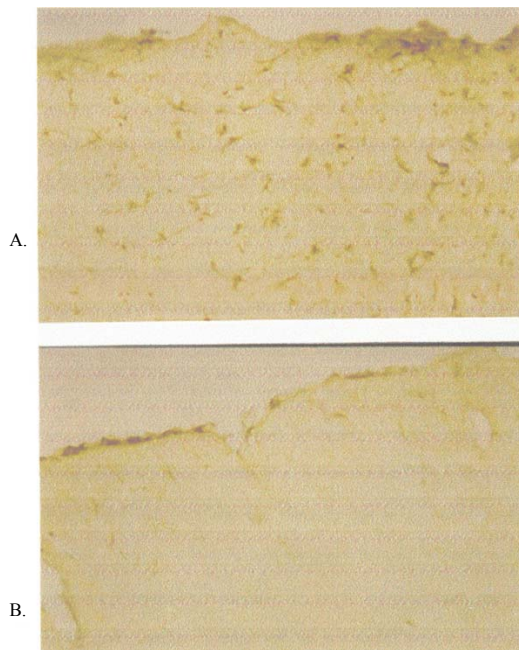


**Fig. 2 – Immunohistochemical staining for cytokeratin 19, ABC method and contrast staining for krezyl-violet.**

A) Numerous cytokeratin 19–positive cells in the right brain hemisphere 7 days after C6 cells implantation ( $\times 400$ ). B) Individual cytokeratin 19–positive cells in the counter lateral hemisphere indicate that C6 cells infiltrated the entire brain ( $\times 400$ ).

implanted, than in the left hemisphere, where they arrived by means of migration. In the control brain sections of normal rats, CK 19 positive cells were found only in the capillary endothelium.

Immunohistochemical reaction with anti-GFAP antibodies revealed numerous GFAP positive astrocytes that were found close to the puncture lesion spot. These astrocytes showed traits of reactive glia, cells swelling, increase in the number and length of extensions (Figure 3).



**Fig. 3 – Immunohistochemical staining for glial fibrillary acid protein (GFAP), ABC method.**

A) Numerous reactive GFAP-positive astrocytes close to the point of C6 cells implantation (× 400). B) Counter lateral hemisphere – only a small number of normal astrocytes were noticed (× 400).

Implanted C6 cells were not GFAP positive. *In vitro*, C6 cells also did not contain GFAP marker of mature glial phenotype.

### Discussion

This study examined experimental brain tumors macroscopically, histologically and immunohistochemically for the presence of proliferation (PCNA) and differentiations (GFAP and CK 19) cell markers.

The study used animal model of primary brain tumor. C6 rat glioma cells are commonly used as glioma cells model for *in vitro* and *in vivo* researches related to tumor cells biology<sup>12</sup>. The C6 glioma cell line has originally been induced on Wistar rats bred by random mating, by means of exposing them to N,N'-nitro-methylurea<sup>2</sup>; once injected into rat brain<sup>13</sup>, it proved by its attributes to be morphologically similar to human malignant glioma. A glioma, a tumor on central nervous system that arise from glial cells, primarily occurs in the brain, and comprise more than 70% of all brain tumors. They are histologically malignant<sup>14</sup> and their typical hallmark is cell proliferation<sup>15</sup>.

It is considered that C6 glioma is analogous to human glioma of II/III malignancy degree. This study used animals in which tumor had been developing for 21–25 days, unlike other authors who used this model, however with tumor development lasting for 10–11 days<sup>16</sup>, 14 days<sup>17</sup>, or 11–21 days<sup>18</sup> following the cerebral implantation of C6 cells.

While measuring the left and the right hemispheres of brains with implanted tumor, a difference was observed in weights of the left and the right hemispheres on the day 7 of tumorigenesis, indicating that glioma cells display invasive ability during active division of glioma cells.

As a marker of cell proliferation, PCNA was monitored immunohistochemically in rat brain having implanted tumor. It was observed that numerous PCNA positive cells were located not only close to the spot of C6 cells implementation, but also in the opposite hemisphere, which supports the migration of C6 cells and their infiltration throughout the brain.

The C6 cells are known to migrate away from the site of implantation and infiltrate the adjacent regions of the brain<sup>10</sup>. Quantification of immunohistochemically stained cells was not possible, given the huge number of implanted cells (4 million), so that monitoring of the increase in number of cells during tumorigenesis was not possible. However, as soon as 7 days after implementation of C6 cells, a large number of PCNA positive cells could be observed in both brain hemispheres, which indicating their intensive proliferation.

Immunohistochemical staining for CK 19, an intermediate filament found in C6 cells *in vitro*, also showed numerous positive cells not only at the place of C6 cells implantation, but also in the opposite hemisphere. The control sections of normal brain showed no CK 19 positive glial cells or neurons.

Previous analysis of histological preparations to GFAP revealed a similar morphology of C6 rat glioma and human glioblastoma, formation of glial edge at the glioma periphery, consisting of GFAP-positive reactive astrocytes. Astrogliosis was monitored until death of animal (28th day)<sup>19</sup>, (30th day)<sup>20</sup>. Reactive astrocytes with multiple processes encircled not only the primary focus of glioma but also any other place of tumor invasion into the nerve tissue<sup>19,20</sup>.

In addition, the injection of radio-tagged monoclonal antibodies on GFAP model of C6 glioma on rats has indicated their accumulation in tumors. Concentration of antibodies was considerably higher in the tumor-affected hemisphere when compared with the unaffected hemisphere<sup>21</sup>.

In this study, the immunohistochemical determination of GFAP revealed that tumor cells do not contain this marker of mature glial phenotype. The C6 cells in culture are also characterized by the absence of GFAP<sup>1</sup>, and they also retain this property after implantation and inducing of brain tumor *in vivo*. Having that said, around the area of puncture lesion inflicted during implantation of C6 cells were discernable numerous reactive astrocytes, which were particularly GFAP positive.

Within the experimental conditions of this study, C6 glioma did not reveal any noteworthy deviations in terms of development, clinical symptomatology and macroscopic anatomy other than those already described in literature<sup>2,22</sup>.

## Conclusion

Macroscopic analysis of C6 rat glioma revealed invasive ability of glioma cells displayed during active division of glioma cells. Histological preparations of rat brain with C6 glioma clearly displayed puncture lesion in the right brain hemisphere, but the tumor mass could rarely be noticed. Microscopic analysis of C6 rat glioma showed the tumor to be of cellular type, with distinct polymorphism of

cells and frequent hyperchromasia of nucleus. Cell proliferation marker PCNA was identified immunohistochemically in C6 cells in both hemispheres of the brain, indicating the proliferative and invasive potential of cells. C6 cells positive to CK 19 were found in both hemispheres of the brain, thus further supporting migration of these cells. Numerous GFAP positive astrocytes were found around the place of puncture lesion. Implanted cells were not GFAP positive.

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